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Forward Genetics: Histidine Auxotrophs in Saccharomyces cerevisiae

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ABSTRACT

Histidine is a crucial amino acid for growth in *S. cerevisiae* and plays other important roles for survival in larger eukaryotes. Histidine auxotrophs, organisms that cannot synthesize histidine, often exhibit inhibited growth and development. Research of the model organism, *Saccharomyces cerevisiae,* illuminates eukaryotic uses of amino acids in other eukaryotes. Researchers have also begun to use histidine auxotrophs as a tag in bacterial or expression vectors. However to perform research with histidine auxotrophs, researchers must isolate them from wild type phenotypes. Our study demonstrated one technique to search for these histidine auxotrophs and it may be vital for the years to come in histidine pathway or expression vector research. Our experiment brings to light the simplicity behind much of today's complex research by taking a basic approach to mutagenesis and selection for mutants.

INTRODUCTION

Saccharomyces cerevisiae, common baker's yeast, has been revered as an excellent model organism for eukaryotes. *S. cerevisiae,* a unicellular, haploid organism is easy to care for and has a rapid generational turnover rate. Research with it allows geneticists to discover the necessary functions, such as the use of amino acids, in eukaryotes. Amino acids provide the building blocks for protein synthesis in eukaryotes and are essential for growth and development. Auxotrophs are organisms that have a genetic mutation that does not allow for them to synthesize certain amino acids. A study on tryptophan auxotrophs who were treated with PHS (phytosphingosine), an inhibitor of tryptophan transport, noted limited growth of the auxotrophs compared to wild type *S. cerevisiae* (Skrzypek 1997).

Histidine, like tryptophan, is an amino acid essential for protein synthesis. *S. cerevisiae* auxotrophs that cannot synthesize histidine may absorb it from their environment to sustain their growth, but growth may still be hindered as exemplified by Skryzpek's study with tryptophan

auxotrophs (1997). Larger eukaryotes that cannot synthesize histidine, but can still obtain it from their food sources may experience other problems aside from inhibited growth. In humans, histidine is used to synthesize histamine and carnosine with the help of the enzyme histidine carboxylase (Fitzpatrick 1982). Histamine is a crucial protein for the immune system in humans as it may act as a neurotransmitter to signal for an inflammatory response (Owen 1987). Organisms that must rely on the environment for histidine may not only display inhibited growth, but inhibited defense responses as well.

Thus research on histidine auxotrophs at the level of simple model organisms, like *S. cerevisiae,* are relevant to larger organisms as well. However it is difficult to conduct research on such mutant organisms when mutations rarely occur unless mutagenized. Furthermore, mutant populations must be screened for the organisms with the specifically desired mutation of auxotrophy. The latter complication is often completed in an enrichment procedure. With *S. cerevisiae,* this can be done by changing growth media several times to stall the development of the histidine auxotrophs and then selecting for a different media that kills all wild type and non-histidine mutants who are dividing and growing.

Our study mutated *S. cerevisiae* with a known mutagen, methyl methanesulfonate (MMS). Later following an enrichment procedure, we screened for histidine auxotrophs amongst the general mutant population. We predicted a high rate of mutagenesis by MMS and a high efficiency of our enrichment procedure when selecting for histidine auxotrophs. We expected to see a much higher rate of histidine auxotroph frequency after enrichment.

MATERIALS AND METHODS

Growing S. cerevisiae with ino1 Mutation

The instructor grew the screening strain (JV01) of *S. cerevisiae* (wild type with an *ino1* mutation) in YPD overnight. To complete this, a colony of the JVO1 plate was scraped with a sterile applicator stick and resuspended in 3.0 mLs of YPD. The colony grew overnight in an incubator with shaking at 30°C. The following day, we determined cell concentration by measuring the absorbance of a 1:10 dilution (300μL of cell culture + 2.7 mL H_2O) of the cell culture at 660nm (A_{660}) with a spectrophotometer . We extrapolated undiluted cell concentration by multiplying the dilute absorbance by 10 and comparing the calculated undiluted cell concentration to a table of cell density where 1.0 mL of cell culture contained 1.1 x 10^7 cells.

Mutagenesis

We split the yeast culture by adding 1mL of the *S. cerevisiae* in YPD to two separate sterile Eppendorf tubes. To the mutant Eppendorf tube, we added 3 μL of methyl methanesulfonate (MMS) and inverted in several times to mix. Both control and mutant cultures were then placed in a test tube rack and incubated for thirty minutes with shaking at 30°C. After incubation, we spun the tubes in a microcentrifuge at max speed for 30 seconds. We then began to wash the cultures of YPD to switch the medium to SD+His to have a more purified controllable medium. To do so, we removed the supernatant, added 1.0 mL of SD+HIStidine medium to each tube, and resuspended the pellet by vortexing it. The residual YPD and MMS were washed away by spinning the tubes at max speed in a microcentrifuge, removing the supernatant, and resuspending the pellet in 1.0 mL of SD+HIS media. We repeated the wash twice to ensure complete removal of MMS and YPD.

We determined the percent survival of the mutagenesis by plating enough of each washed culture to grow 100 and 500 colonies per plate. This required us to complete a serial dilution of the cell culture (Table 1). Plates were incubated at 30°C for three days. We determined percent survival of mutagenesis by dividing the amount of colonies on the mutagenized plate by the amount of colonies on the control plate.

Table 1. Volumes used for % survival plating

	Control	Mutant
100 cells/plate	205 mL of 1: 10^5 dilution	205 mL of 1: 10^5 dilution
500 cells/plate	102 mL of 1: 10^4 dilution	102 mL of 1: 10^4 dilution

To a sterile test tube designated for the mutant culture we added 4.5 mL of SD+HIS medium and 0.5 mL of the washed mutagenized culture. Conversely, to a sterile test tube designated for the control culture we added 4.95 mL of SD+HIS medium and 0.05 mL of the washed control culture. Both cultures were allowed to recover from mutagenesis by incubating them with shaking at 30°C overnight. We limited further growth of the cultures by keeping them in a refrigerator until one day prior to enrichment. We woke up the cultures by resuspending them via vortex and transferring 1mL of each culture to separate sterile test tubes containing 4.0 mL of SD+HIS media. Both cultures were then incubated at 30°C with shaking overnight until they could be used for enrichment.

Enrichment of Histidine Auxotrophs

8 hours prior to enrichment we transferred 1.0 mL of the overnight cultures to separate Eppendorf tubes and spun the tubes at max speed in a microcentrifuge for 15 seconds. We then removed the supernatant and resuspended the pellet in 1.0 mL of SD media. This wash of SD+HIS with SD-His media was repeated twice more. To two sterile test tubes containing 4.5

mLs of SD-His media, we added 0.5 mL of each washed culture to its own tube. We allowed the cultures to grow by incubating them at 30°C with shaking for eight hours. Switching the medium from SD+His to SD-His allowed the control culture to grow and to halt growth in the mutagenized culture.

By creating a 1:6 dilution of each culture (0.5 mL of cell culture + 2.5 mL of SD), we determined cell concentration through absorption (A_{660}) with a spectrophotometer . We calculated cell concentration through absorption by using a table of cell density where 1.0 mL of cell culture contained 1.1 x 10^7 cells.

The remaining cultures were transferred to Oakridge tubes and spun for five minutes in the SS34 rotor at 15,000 rpm. After spinning the samples, we removed 4.0 mLs of the supernatant, resuspended the pellet by vortexing it, and transferred the concentrated samples to separate Eppendorf tubes. We recollected the pellets by spinning them in a microcentrifuge at max speed for 15 seconds. We began washing the culture by removing the supernatant, adding 1 mL SD-INO to the tubes, vortexing the cultures and repeating microcentrifugation to recollect the washed pellet. We repeated the washing process twice more for a total of 3 washes. To two separate sterile test tubes, we added 4.0 mLs of SD-INO media and the entirety of the washed culture to its respective tube. Both cultures were then incubated overnight at 30°C with shaking. We switched the medium from SD-His to SD-INO to kill off cells that were growing. Histidine auxotrophs would have stopped growing from the time spent in SD-His and thus would have survived the starvation period in SD-INO.

The following day we began screening for histidine auxotrophs. We plated enough of each culture to grow 50 and 250 colonies per plate (Table 2) while acknowledging a 99% killing rate that occurred during enrichment. We grew the colonies in an incubator for three days at

30°C after which we moved the plates to a refrigerator until replica plating. The growth of the remaining enrichment culture stocks was limited by keeping the cultures in a refrigerator. We calculated the efficiency of enrichment by comparing the amount of living cells per mL after enrichment to the amount of living cells per mL pre-enrichment.

Table 2. Volumes Used for Enrichment

	Control	Mutant
50 cells/plate	65.9 μL of $1:10^3$ dilution	151 μL of $1:10^1$ dilution
250 cells/plate	328.8 μL of $1:10^3$ dilution	75.7 μL of full strength

Selection of Histidine Auxotrophs: Replica Plating

We began our selection of histidine auxotrophs by creating YPD and YC-HIS replica plates of the both cultures' 50 cells/plate plates. We incubated the replica plates for three days at 30°C and then moved them to a refrigerator until we could begin confirmation of our histidine auxotroph findings.

Confirming the Identity of Histidine Auxotrophs

We refined our selection of histidine auxotrophs by selecting colonies from the replica plates that could grow on the YPD medium plates, but not the YC-HIS medium. These colonies were streaked onto new YPD and YC-HIS plates with a wild type sample from the control. We allowed the cultures to grow in an incubator for 2 days at 30°C and photographed our results.

From our findings, we determined the mutation frequency of histidine auxotrophs. We did so by comparing the number of mutants in the control to the mutagenized culture. We also calculated the factor by which enrichment increased mutant frequency by comparing mutant frequency before and after enrichment.

RESULTS

Mutagenesis

Through spectrophotometery, we determined the concentration of both the original mutant and control cultures to be 4.895 x 10^7 cells/mL (Table 3). This value allowed for us to calculate the volume used to plate the mutagenesis efficiency plates and thus determine mutagenesis efficiency. The control plate plated with enough culture to grow 500 cells/plate grew more than 500 colonies and thus both the mutant and control plates of 500 cells/plate were disregarded for accuracy reasons. We observed a significant amount of mutagenesis in our plates plated for 150 cells/plate (Table 4).

Table 3. Percent Survival Throughout Experiment

	Prior to Mutagenesis	After Mutagenesis	Survival	Prior to Enrichment	After Enrichment	Survival
	(cells/mL)		(%)	(cells/mL)		(%)
Control	4.895 x 10^7*	7.59 x 10^6	-	7.59 x 10^6	1.48 x 10^7	195.13
Mutant	4.895 x 10^7	3.30 x 10^5	4.67	3.30 x 10^5	1.92 x 10^3	0.58

* Cell concentration determined through spectrophotometry

Table 4. Mutagenesis Efficiency

	Control	Mutant	% Survival
100 cells/plate	150	7	4.67
500 cells/plate	Lawn growth observed, calculations disregarded		

Enrichment

Spectrophotometry also allowed for us to discover the cell concentration after mutagenesis of the control and mutant cultures (Table 3). The control culture was far more concentrated than the mutagenized culture. We took this into account when plating for efficacy

of enrichment. As expected, we observed a killing rate of about 99% as we saw only 0.58% of the mutants survive (Table 3 and Table 5).

Table 5. Determination of Efficacy of Enrichment

Plate	Total Number of Colonies	Volume Plated (mL)	Dilution Factor	Number of Live Cells/ mL		% Survival of Enrichment
				Post-Enrichment	Pre-Enrichment	
Mutant	29	151.0	10^1	1.92×10^3	3.30×10^5	0.58
Control	976	65.9	10^3	1.48×10^7	7.59×10^6	195.13

Mutagenesis Frequency

Initially from our replica plates, we observed four possible mutants out of twenty-nine colonies (mutation frequency post-enrichment= 13.8%). However, when we streaked these colonies for a secondary assessment, we observed only one colony that could grow on YPD plates, but not YC-His plates (Figure 1). We concluded our study of *S. cerevisiae* histidine auxotrophs demonstrated a 3.4% mutagenesis frequency. Enrichment greatly increased our finding of His-mutants by a factor of 170 (Table 6). Prior to enrichment we observed 0.02% mutant frequency, however after enrichment; we observed 3.4% mutant frequency (Table 6).

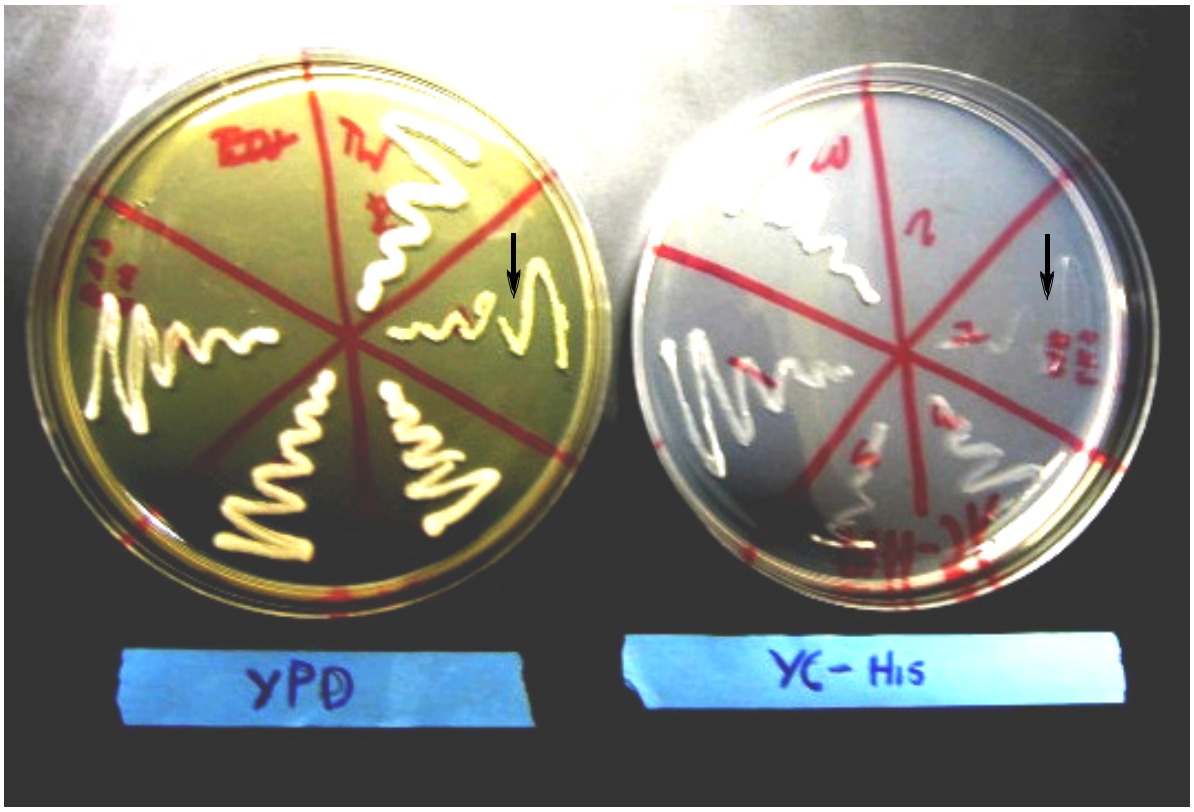

Figure 1. Confirming the identity of histidine auxotrophs

Four suspected mutant colonies and one wild type colonies were tested for their ability to grow on YPD, but not YC-His plates. Three of the four colonies grew on both plates suggesting wild type characteristics, but the one mutant colony (labeled with arrows) could not grow on the YC-His plate.

Table 6. Efficacy of Enrichment

	# Surviving After Enrichment			# Surviving Before Enrichment			
	All Cells	His- Mutants	His- Frequency (%)	All Cells	His- Mutants	His- Frequency (%)	Factor
Control	1.48×10^6	0	0	7.59×10^6	0	0	0
Mutant	1.92×10^3	66.21	3.4	3.3×10^5	66.21	0.02	170

DISCUSSION

Our data supported our hypothesis as our forward genetics experiment demonstrated a high rate of mutagenesis of *S. cerevisiae* using MMS. The high rate of mutagenesis increased our odds for selecting histidine auxotrophs. This selection was further refined using a highly efficient enrichment procedure that increased the frequency His-mutants by a factor of 170. In conclusion, our study practices an excellent technique for the mutagenesis and selection of histidine auxotrophs.

There are several caveats to our study that require further analysis. The one of most concern centers around the fact that after enrichment the control culture had a survival rate of 195.13% which implies the population almost doubled during enrichment. This could be explained by human error. It could be possible we plated too much of the control culture than we calculated for or there may have been an error when making the serial dilution. Both errors would reflect in possible growth of populations.

Our experiment still lays the fertile soil for future research in both the direction of histidine auxotrophs and efficacy of enrichment. Our study demonstrated the astounding effects of enrichment as we had a 170 fold increase in mutation frequency in our mutagenized sample. Enrichment clearly allows for the true results of a study to shine through more easily by concentrating the desired mutant within a culture. However, enrichment can also be applied in a variety of techniques and may help uncover rare and subtle genetic characteristics. Gardner *et al.*, using a modified enrichment procedure based on the magnetic and biotin capture of repeating sequences, isolated and identified more than ten tetranucelotide sequences in a social lizard, *Egernia stokesii* (1990). These tetranucleotide sequences can then be used in future research to determine evolutionary links amongst *E. stokesii* and other lizards. Thus further research that

expands enrichment techniques may help solve dilemmas on a grander scale of big biology as it can help refine phylogenetic trees with a subtle genetic touch.

Research of histidine auxotrophs illuminates the prevalence of certain bacterial ailments. Juliao *et al.* showed histidine is detrimental to bacterial growth and survival of *Haemophilus influenza* (2007). Otitis media lacking histidine allowed for limited growth and survival of *H. influenza* while throat media rich with histidine and other nutrients allowed for the auxotroph bacteria to flourish. Additionally, future research of histidine auxotrophs opens the door to tagging expression vectors. Expression vectors create heterologous DNA by inserting specific genes in with a plasmid. Often times, the plasmids are tagged with histidine auxotrophic characteristics when the desired characteristic of the plasmid does not have an obvious or apparent phenotype. Thus by growing the samples on medium lacking histidine, researchers can monitor integration of the plasmids by survival of the heterogenous samples. Samples with greater integration will survive in greater proportions compared to samples with little plasmid integration (Pronk 2002). Our study demonstrated one technique to search for these histidine auxotrophs and it may be vital for the years to come in bacterial or expression vector research. Our experiment brings to light the simplicity behind much of today's complex research by taking a basic approach to mutagenesis and selection for mutants.

WORKS CITED

Fitzpatrick, D. W., Fisher, H. 1982. Histamine synthesis, imidazole dipeptides, and wound healing. Surgery **91:**430-434.

Gardner, M. G., S. B. Cooper, C. M. Bull, W. N. Grant. 1990. Brief communication: Isolation of microsatellite loci from a social lizard, *Egernia stokesii*, using a modified enrichment procedure. The Journal of Heredity **90:** 301-304.

Juliao, P. C., C. F. Marrs, J. Xie, J. R. Gildorf. 2007. Histidine auxotrophy in commensal and disease-causing nontypeable *Haemophilus influenzae.* Journal of Bacteriology **189**:4994-5001.

Owen, D. A. 1987. Inflammation---histamine and 5-hydroxytryptamine. British Medical Bulletin **43:** 256-269.

Pronk, J. T. 2002. Auxotrophic yeast strains in fundamental and applied research. Applied and Environmental Microbiology **68:** 2095-2100.